High Throughput Screening in Drug Discovery for Cancer Research Peptides Using High Resolution LCMS

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Overview

Purpose: To implement high resolution accurate mass detection for the simultaneous analysis of multiple components in a high throughput screening assay.

Methods: Several peptides commonly monitored for cancer research were analyzed at various concentration levels in HEPES buffer solutions to determine signal response, linearity, reproducibility, and Z' value.

Results: Full scan mode at a resolving setting of 70,000 was utilized for the analysis of several thousand sample sets at cycle time of 30 seconds per sample. Four individual peptides were simultaneously analyzed with results demonstrating a linear signal response across the working range of the assay with analyte sensitivity in the low nM range. Full Scan operation at 70,000 resolving power enabled for data collection across a wide mass range allowing for multiple peptide monitoring at multiple charge states and detection of unexpected sample changes with no impact on instrument performance.

Introduction

Lead discovery groups are constantly developing new methodologies to screen large chemical libraries against a specific target. The majority of these methods utilize plate reader devices, ELISA, and other analytical techniques that are amenable to high throughput analysis. Existing analytical methodology used for screening can be less than ideal due to sample prep considerations, limit of detection, and specificity of analyte detection. In these cases high resolution LCMS can provide a high quality alternative while maintaining a sampling rate that is suitable for HTS requirements. Liquid chromatography allows for analysis of samples in matrices that otherwise cause interference in the MS source, while high resolution MS provides sensitivity and specificity along with ease of use without compromising data quality. Two commercially available peptide sets commonly targeted for cancer research were analyzed in a simulated enzymatic screening assay experiment. Survivin 2B and Bid BH3 which are often targeted for analysis for their role in cell apoptosis were monitored at various concentrations in a high throughput screening experiment and the results analyzed to determine signal response, linearity, reproducibility, and Z' value.

Methods

Sample Preparation

Five lyophilized peptides were ordered (catalog numbers: Survivin 2B (80-88) cat# 62693, Survivin (85-93) cat# 62691, Bid-BH3 cat# 61631, Bid BH3 (85-98) cat# 62485 and Substance P cat# 61694 from AnaSpec™, San Jose, CA). Each peptide was reconstituted in a deionized water at 1 mg/mL. Serial dilutions of Survivin and Bid-BH3 were prepared at concentrations of 1 nM, 5 nM, 10nM, 100 nM, and 1000 nM in in a 50 mM HEPES solution containing 50 mM MgCl2, 50mM Citric Acid, Tween20, and 1% (v/v) DMSO). Substance P was then added to each dilution level as an internal standard at a final concentration of 100 nM.

Liquid Chromatography and Multiplexing

Samples were injected onto a C18, 42.1 x 30 mm, 3.5 µm HPLC column. Analyte elution was accomplished using mobile phase A - water + 0.1% Formic Acid (v/v) and mobile phase B - Acetonitrile + 0.1% Formic Acid (v/v) with a 1-minute step gradient at a flow rate of 1.2 mL/min. The step gradient was performed at 0-10 sec, 2% B, 11-35 sec, 35%B, 36-65 sec, 95% B, and 66-100 sec, 5% B. All methods were completed using a Thermo Scientific Transcend LX-4 system with a dual injector arm with DLW (Dynamic Load and Wash). LC multiplexing was implemented with a data window of 18 seconds per LC channel.

Mass Spectrometry

A Thermo Scientific Exactive Plus bench-top Orbitrap mass spectrometer (Figure 1) was used in Full Scan and Full Scan all-ions-fragmentation (AIF). Full Scan data was collected across a mass range of 350-615 m/z at resolution 70,000 and a spectral speed of 3 Hz. A maximum inject time of 250ms for the Full Scan was applied. Full Scan AIF was collected with Full Scan across a mass range of 350-615 m/z at resolution 70,000 and a spectral speed of 3 Hz, and Full Scan AIF across a range of 200 – 2000 m/z at resolution of 17,500 and a spectral speed of 12 Hz using a Normalized Collision Energy of 25. A maximum inject time of 250ms was applied for the Full Scan with 50ms applied for the Full Scan AIF.

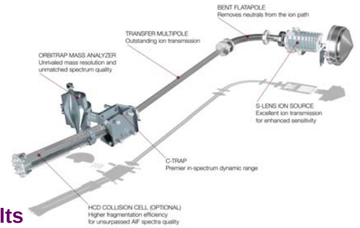
Ion source conditions were consistent for all MS experiment scan modes with the following parameters held constant, Vaporizer temperature 500°C, capillary temperature 375°C, sheath gas 60, and AUX gas 20. Prior to experimental data collection, the instrument was calibrated in positive ion mode using Pierce LTQ Velos ESI Positive Ion Calibration Solution.

In all runs, multiple sample injections were collected into a single data file format using Thermo Scientific Xcalibur and Aria software to automatically control data acquisition start and stop times for each sample set. Commercially available peptides were analyzed at various concentration levels and over multiple replicates to determine the coefficient of variation, linear response, and lower limit of quantitation. Results for each experiment type were compared for overall performance and ease of use.

Data Analysis Software

Thermo Scientific Pinpoint software was used for exact mass calculation of all peptides at each of the observable charge states. Exact mass information was saved in Microsoft® Excel® for later use. Thermo Scientific QuickCalc software (powered by Gubbs Inc., GMSU Gubbs™ Mass Spec Utilities, Atlanta, GA) was used for all chromatographic data review, and report generation. Compound information was imported into QuickCalc™ software using an Excel import template containing the required *m/z* information. The compound information was stored in QuickCalc software for later use. The compound information was then grouped into a "compound set" and saved, allowing the exact *m/z* information for multiple compounds to be easily recalled and applied to an entire data set for easy chromatographic review. QuickCalc software was also used to automatically extract and sort each of the 96 injections contained in a data file and then display individual chromatographic peaks in a table view correlated with all sample information related to each individual injection.

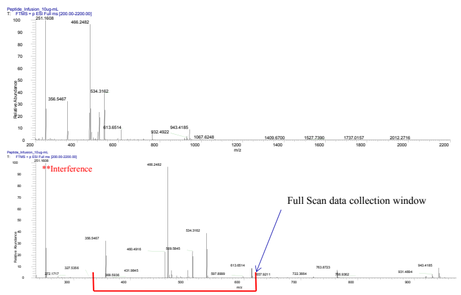
FIGURE 1. Exactive Plus Instrument Schematic



Results

Theoretical exact mass calculations were confirmed for each peptide by directly infusing with 1 µg/mL solution of each compound at 5 µL/min. (Figure 2) The charge states producing the most abundant signal for each peptide were determined during the infusion procedure and the mass range for the full scan experiment set accordingly to monitor all relevant charge states. Peptide charge states producing a low level response were excluded from the data collection window to reduce overall data file size, with all charge state ions below 10% of the total signal excluded from the collection range. Files containing 96 or more injections were limited to an overall size of less than 500MB.

FIGURE 2. Top: Full scan peptide mix from 200 to 2000m/z. Bottom: zoomed scan range from 200 to 950m/z.

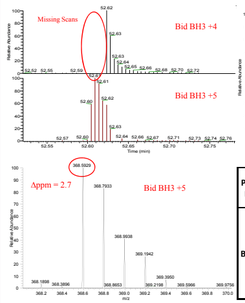


Samples in HEPES Buffer solution were analyzed at 5 concentration points ranging from 1nM to 1000nM with 288 replicate injections at each concentration level. The average peak area ratio was used to calculate %CV at each concentration and generate a calibration curve. (Table 1) The LOQ was determined to be 1nM for three peptides and 5nM for the fourth, with a linear signal response across the detected concentration range. Three of the peptides demonstrated a linear and reproducible response with %CVs below 20% at all concentration levels. The fourth peptide, although detectable at all concentration points displayed %CVs above 20% even at increasing concentration levels. Z-prime statistical analysis provides an indication of overall assay robustness based on the average and standard deviation of a positive and negative control. Z-prime values above 0.5 indicate a robust and acceptable assay. The z-prime for the forth peptide was well below 0.5 with a value of 0.32, indicating a lack of robustness in the analysis. Upon subsequent examination an unresolved interference was observed for the m/z of the fourth peptide that was not present during assay method development. The m/z collection range utilized in the full scan MS experiment allowed for reanalysis of the peptide at an alternate charge that provided an acceptable but lower signal response. The average, standard deviation, %CV, and z-prime was recalculated for the fourth peptide at the alternate charge state through re-interrogation of the previously collect data. (Figure 3)

Table 1. Mean, standard deviation, %CV and z-prime for each peptide analyzed. Highlighted values indicate %CV and z-prime values above acceptable levels.

Peptide Name		Conc (nM)				
		1	5	10	100	1000
Survivin 85-93	Avg.	0.012532	0.063424	0.135694	1.435063	12.96369
	Std Dev	0.001733	0.007772	0.011968	0.16085	0.562784
	%CV	13.82914	12.25476	8.820187	11.20856	4.34123
	z-prime			**0.87**		
		1	5	10	100	1000
Survivin 2B 80-88	Avg.	--	0.005237	0.01118	0.133545	1.28023
	Std Dev	--	0.000832	0.001688	0.019558	0.126222
	%CV	--	15.87972	15.0943	14.64532	9.859341
	z-prime			**0.71**		
		1	5	10	100	1000
Bid BH3 85-98	Avg.	0.00490	0.02708	0.05928	0.78428	7.52874
	Std Dev	0.00094	0.004446	0.007849	0.097311	0.380001
	%CV	19.18882	16.41506	13.24001	12.4077	5.047339
	z-prime			**0.81**		
		1	5	10	100	1000
Bid BH3	Avg.	0.01718	0.04581	0.08430	0.62186	5.64208
	Std Dev	0.003777	0.007009	0.01349	0.20801	2.483671
	%CV	21.98924	15.30206	16.00155	33.44948	44.0205
	z-prime			**0.32**		

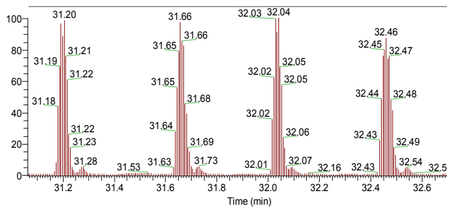
FIGURE 3. Reanalysis of Bid BH3. (Top Left) Bid BH3 scans at +4 and +5, missing scans indicate deviation outside of 5ppm analysis window. (Top Right) MS spectra at +4, Δppm = 7.1 indicate an unresolved interference. (Bottom Left) MS spectra at +5, Δppm = 2.7. (Bottom Right) Table of recalculated Avg, Std. Dev., %CV, and z-prime.



Peptide Name		Conc (nM)				
		1	5	10	100	1000
Bid BH3	Avg.	--	0.00039	0.00097	0.01210	0.11674
	Std Dev	--	7.1E-05	0.000163	0.002023	0.012911
	%CV		18.11	16.75	16.72451	11.05993
	z-prime			**0.61**		

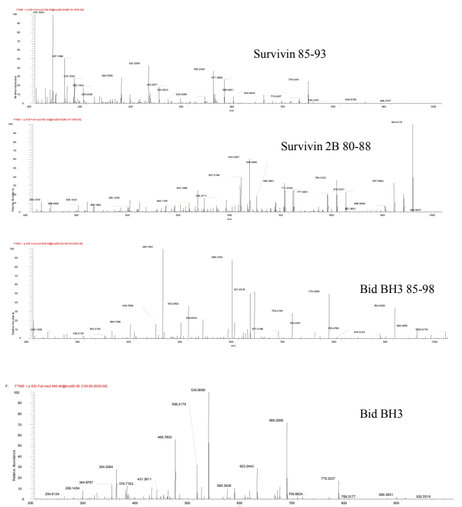
Sample sets were also collected using All Ion Fragment (AIF) to collect fragmentation spectra for all peptides present in the sample injection. The Full Scan was collected at a resolving power of 70,000 at 200m/z while the AIF was collected at a resolving power of 17,500 at 200m/z. The scans at the resolving power specified above allow for an average of 30 scans across the six second wide chromatographic peak. (Figure 4)

FIGURE 4. AIF collection at 17,500 from 200-1000m/z. Scan settings provide for an average of 30 scans across the 6 second peak.



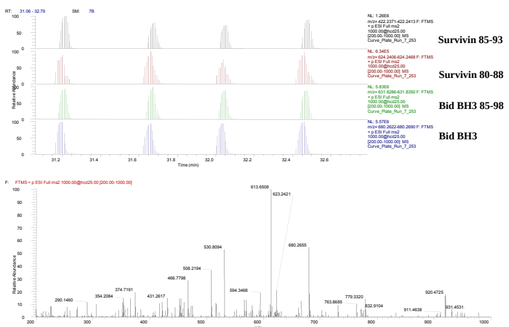
Each peptide analyzed was interrogated for possible fragmentation prior to LC analysis. Neat solutions of each peptide were individually infused to determine the peptide fragmentation pattern. (Figure 5)

Figure 5. Infusion of neat peptide solutions at 10uM conc. and resulting MSMS spectra



The fragmentation data collected during infusion was used to analyze AIF data collected during Full Scan analysis. The most intense fragmentations for each peptide were used to generate extracted ion chromatograms for the AIF data. The results demonstrate the presence of the expected fragmentation patterns for all peptides with scan speed, peak shape, retention time, and sensitivity adequate for MSMS confirmation as well accurate quantitation. The addition of the AIF did not affect the quantitative results of the Full Scan component of the experiment due to the rapid scanning speed of the MS.

FIGURE 6. MSMS extracted ion chromatograms (XIC) for all four peptide in simultaneous analysis across a four channel multiplexing system. (Top) AIF XIC's for each individual peptide across a four channel system. (Bottom) AIF spectra at chromatographic peak apex.



Conclusion

- Liquid chromatography with high resolution mass spec analysis provided a robust and reproducible method for high throughput screening and simultaneous analysis of multiple peptides at multiple charge states over the course of more that 3000 individual sample injections.
- Full scan data collection across a wide mass range enabled the acquisition of spectral information for the entire isotopic charge envelope without sacrificing data quality.
- Statistical analysis of a large injection set resulted in Z' values ranging from 0.61 to 0.85 indicating method robustness the analysis.
- Multiplexing using a four-LC channel system provided rapid injection cycle times of 30 seconds, on average, while proving separation from detergents and other interferences containing in sample matrixes.
- Full Scan / Full Scan AIF data collection allows for simultaneous Full Scan quantitation as well MS/MS confirmation or quantitation with minimal reduction in instrument scan speed.

References

1. Zhang,J.-H.; Chung,T.D.Y.; Oldenburg,K.R. *J. Biomol. Screening* **1999,** 4, 67-73.
2. Roddy,T.P.; Horvath,C.R.; Stout,S.J. *Anal. Chem.* **2007,** 79, 8207-8213.